One- and two-photon time-resolved fluorescence of visible and near-infrared dyes in scattering media

R. Esposito[a], C. Altucci[a], R. Velotta[a], G.M. Gaeta[b], M. Lepore[c]
[a]*Dipartimento di Scienze Fisiche, Università"Federico II", Unità CNISM, Napoli, Italy*
[b]*Dipartimento di Malattie Odontostomatologiche, Seconda Università di Napoli, Italy*
[c]*Dipartimento di Medicina Sperimentale, Seconda Università di Napoli, Napoli, Italy*

ABSTRACT

Visible and near-infrared dyes are largely used in diagnostics and sensing. For this reason, it is very important to study their time-resolved fluorescence in presence or in absence of proper scattering medium in order to simulate the optical characteristics of biological tissues. Moreover, if one- or two-photon excitation processes are available also visible dyes will be employed taking advantages from using exciting sources in the diagnostic window (red and near IR) of the electromagnetic spectrum, where the photons are rarely absorbed and more often scattered. Visible and near IR fluorescent samples (Indocyanine Green and Rhodamine 6G) in absence and in presence of scattering agents (different Intralipid concentrations) and one- and two- photon time-resolved experiments have been performed. As expected, the presence of scattering agents modified time-resolved spectra and the related lifetime components. The experimental results have been used to preliminarly test different theoretical approaches describing the propagation of fluorescence signals in scattering media.

Keywords: time-resolved fluorescence, infrared and visible dyes, scattering media

1. INTRODUCTION

The interest in using fluorescence in the field of diagnostics is related to the evidence that fluorescence is a process very sensitive to the biochemical variations undergone by tissues. The fluorescence signals can be due to endogenous fluorophores that are naturally present in the tissues or to exogeneous ones that can be artificially injected and give more intense signals. For example, the study of Indocyanine Green, widely used in clinical diagnostics from retinal and choroidal videoangiography to the estimation of the severity of burns is of great interest. In fact, the absorption and the emission of ICG in tissue has been easily detected in the IR part of the so-called diagnostic window. In the last years also the two-photon absorption processes have been largely adopted for exciting visible emitting probes by means of infrared radiation in order to get larger penetration depth with less damage. In the present paper we report the results of one- and two- photon time-resolved fluorescence measurements on visible and near IR fluorescent samples (Indocyanine Green and Rhodamine 6G) in absence and in presence of scattering agents (different Intralipid concentrations). As expected, the presence of scattering agents modified time-resolved spectra and the related lifetime components. The experimental results have been used to preliminarily test different theoretical approaches describing the propagation of fluorescence signals in scattering media.

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2. THEORETICAL BACKGROUND

As well know the intensity of light emitted by a fluorophore as a function of time is given by a sum of decaying exponentials [1]:

$$I(t) = \Sigma_i \, Ai \exp(-t/\tau_i) \qquad (1)$$

The number of terms in the sum (and, consequently, the number of lifetimes τ_i) depends on the number of transitions involved in the fluorescence decay. In the case of ICG, the transition occurs between the first excited singlet state S_1 and the fundamental singlet state S_0: the decay is then mono-exponential. It is well-known that scattering in random media results in change in the apparent in the apparent fluorescent lifetime of dye [2]. In fact, fluorescent lifetime of fluorophores determined in highly scattering media are usually distorted because the "time of flight" associated with the migration of the excitation and emission photons. Also absorption changes can alter photon migration and can distort the measured lifetime. To account for these effects different mathematical models have been developed. For example Patterson et al [3] give an analytical expression for the fluorescence signal generated by a fluorophore distributed in a scattering sample by assuming that the scattering properties of the host medium are the same at the excitation and emission wavelength. In this model the total fluorescence signal per unit time and area is expressed as follows :

$$F_{xm}(r,t) = \frac{\mu_{fx}\Phi_{xm}cz_0}{(4\pi Dc)^{3/2}(\beta_x - \beta_m)} t^{-5/2} \exp\left(-\frac{(\mu^2 + z_0^2)}{4Dct}\right)\left[\exp(-\beta_m t) - \exp(-\beta_x t)\right] \qquad (2)$$

with

$$z_0 = \frac{1}{\mu_s(1-g)}$$

and

$$D \cong \frac{1}{3\mu_s(1-g)}$$

where ρ is the distance between the point where the source light is delivered on the sample and the point where the emitted radiation is detected; μfx is the product of the concentration C of the fluorophore and the extinction coefficient at λx; $\Phi xm = \Phi(\lambda_x, \lambda_m)d\lambda_m$, where $\Phi(\lambda_x, \lambda_m)$ is the quantum efficiency per unit wavelength for emission at λm; g is the scattering anisotropy parameter ; D is the diffusion coefficient; c is the speed of light in the medium; $\beta x = c(\mu_{ax} + \mu_{fx})$ and $\beta m = c(\mu_{am} + \mu_{fm})$. Using the above-reported expression it is possible to extract information on the absorption and reduced scattering coefficients of the scattering media if the optical properties of fluorophore (μfx, μfm and Φxm) are known.

Hattery et al [4] have conceived an analytical model where the expression of the probability of a fluorescence photon to be detected in a certain position and in a certain instant is given by:

$$\gamma(t,r') = \frac{\mu_{af}}{\mu_{si}} \Phi \left[p(t,r') - \langle \Delta t \rangle \frac{dp(t,r')}{dt} \right] \qquad (3)$$

where μ_{af} and μ'_{si} are the probability of absorption by the fluorophore and the transport-corrected scattering coefficient at the excitation wavelength respectively; Φ is the quantum efficiency of the fluorophore; p(t, r') is the probability of a photon arriving at point r' at time t absent any fluorescence behavior; $<\Delta t>$ is the fluorescence delay and coincides with the fluorescence lifetime τ. Therefore, in this model the delay of photons that results from the excitation of and subsequent emission by a fluorophore may be treated as an en route delay that can be isolated from transit delays caused by multiple scattering. The acquisition of an accurate numerical value of the fluorescence lifetime in every single point of the sample is of fundamental importance in the development of image-reconstruction techniques (the so-called lifetime imaging techniques). Then this model could offer a very useful tool for this kind of imaging.

3. MATERIALS AND METHODS

3.1Experimental set-up

The set-up equipped with a Ti: Sa laser and a synchroscan streak camera allows us to have a temporal resolution of few picoseconds. In particular, the system is composed by a Ti:Sa laser with a repetition rate di 76 MHz, a pulse duration of 130 fs, and an average power of 1.5 MW at 800nm (Coherent, Model MIRA 900DUAL) that was pumped by an argon-ion laser (Coherent, Model Sabre 400) and a streak camera (Hamamatsu Photonics, Model 5680, S1-IR extended photocatode). To select the emission wavelength and reject laser light a 90° geometry collection and proper coloured filters have been adopted. Further details on the experimental set-up have been reported in ref. [5]

3.2 Samples preparation

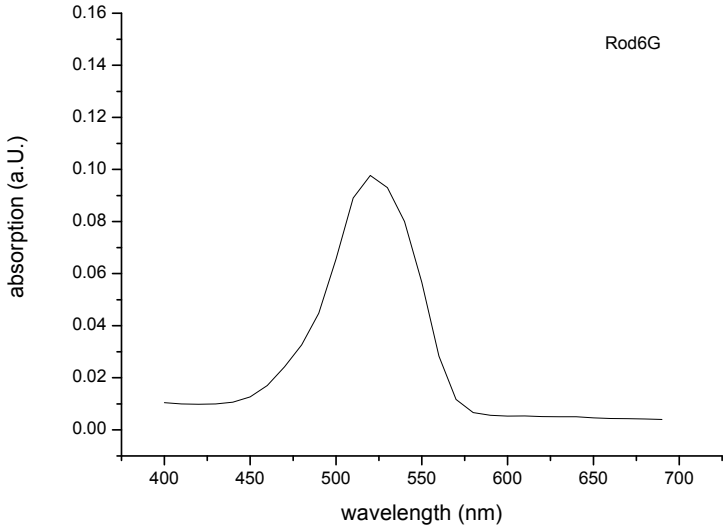
Different ICG and Rod6G solutions with proper solvent (dymetilsulfoxide, ethanol and methanol) have been prepared. Preliminarily absorption and steady-state fluorescence spectra have been measured with conventional spectrophotometer and spectrofluorimeter. Time-resolved fluorescence spectra have been acquired with the experimental set-up above-mentioned. To prepare the scattering samples appropriate quantities of Intralipid 10% have been added to get different concentrations of scattering suspensions. As an example in Table 1 are reported the scattering parameters for ICG

3.3 Data analysis

Different procedures have been adopted in analyzing the time-resolved fluorescence spectra. As a first step the experimental data have been compared with an expression similar to Eq.1 considering a mono-exponential or a bi-exponential decay. The impulse response functions has been properly convolved with the theoretical predictions and the experimental data have been fitted with the convolved expression by minimization of χ^2 parameter. Moreover also Eq. 2 has been adopted for analyzing data of scattering samples. In this case also data obtained by previously reported measurements of reflectance and steady-state fluorescence have been used [6]

4.RESULTS AND DISCUSSION

In fig. 1 typical absorption spectra for Rod 6G and ICG samples have been reported. For Rod 6G sample a maximum has been found at 510 nm and for ICG a maximum has been found at 780 nm. Measurements with samples prepared with different solvent and at different concentrations has shown little changes in the maximum location as expected from literature



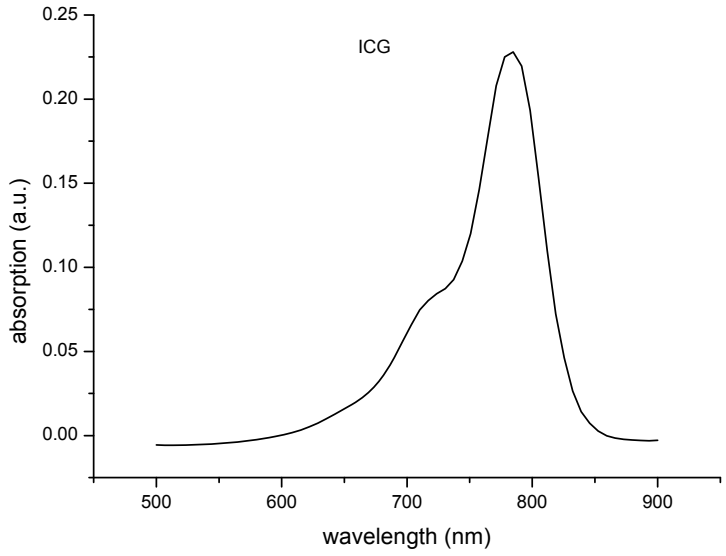
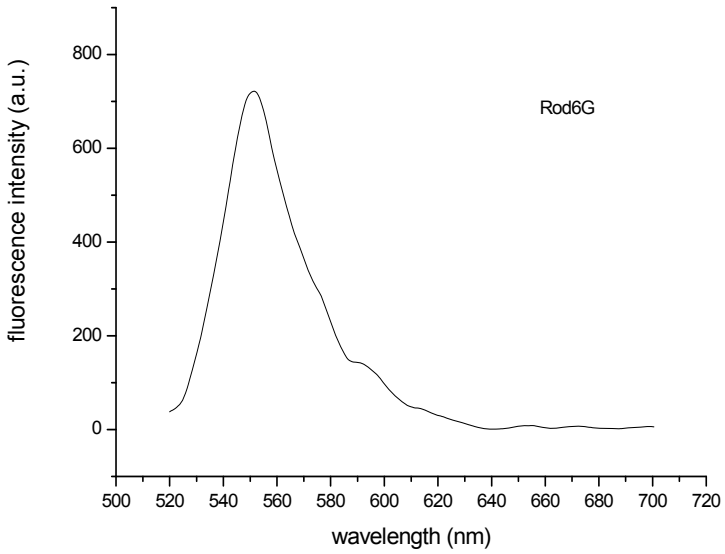


Figure 1. Rod 6G (solvent: methanol; concentration 10^{-6} M) and ICG (solvent: ethanol; concentration:10^{-6} M) absorption spectra.



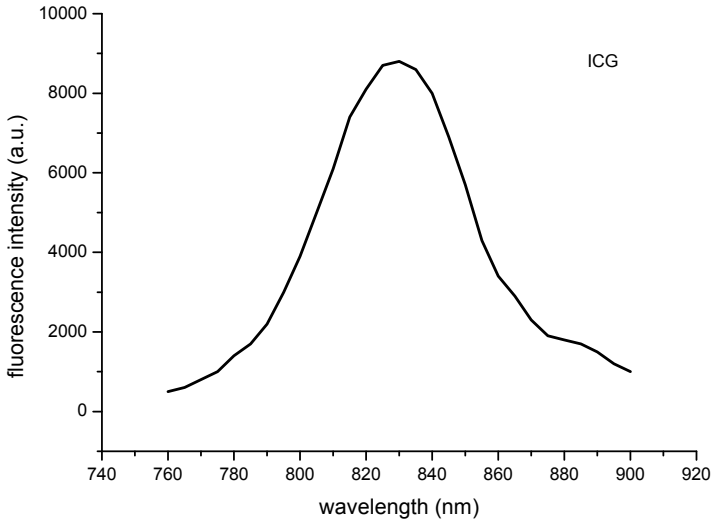


Fig.2 Fluorescence spectra of Rod 6G (concentration = 10^{-6} M, excitation wavelength =510 nm) and ICG (concentration = 10^{-4} M, excitation wavelength = 638 nm).

In fig. 2 typical fluorescence spectra for a Rod 6G and ICG are reported. The former spectrum has been obtained by means of a conventional spectrofluorimeter (LS55 – Perkin Elmer) the second one has required a more

sensitive apparatus equipped with a CCD device for signal detection . In this case the fluorescence has been excited by a He-Ne laser. For Rod 6G sample the fluorescence maximum has been found to be located at 530 nm and the predicted displacements with concentration and solvent has been observed [1]. For ICG samples the maximum is located at 820 nm. All the information obtained from steady-state measurements have been employed in time-resolved fluorescence measurements. In fig. 3 typical time-resolved fluorescence spectra for ICG (linearly excited) and Rod 6G (two-photon excitation processes) have been reported.

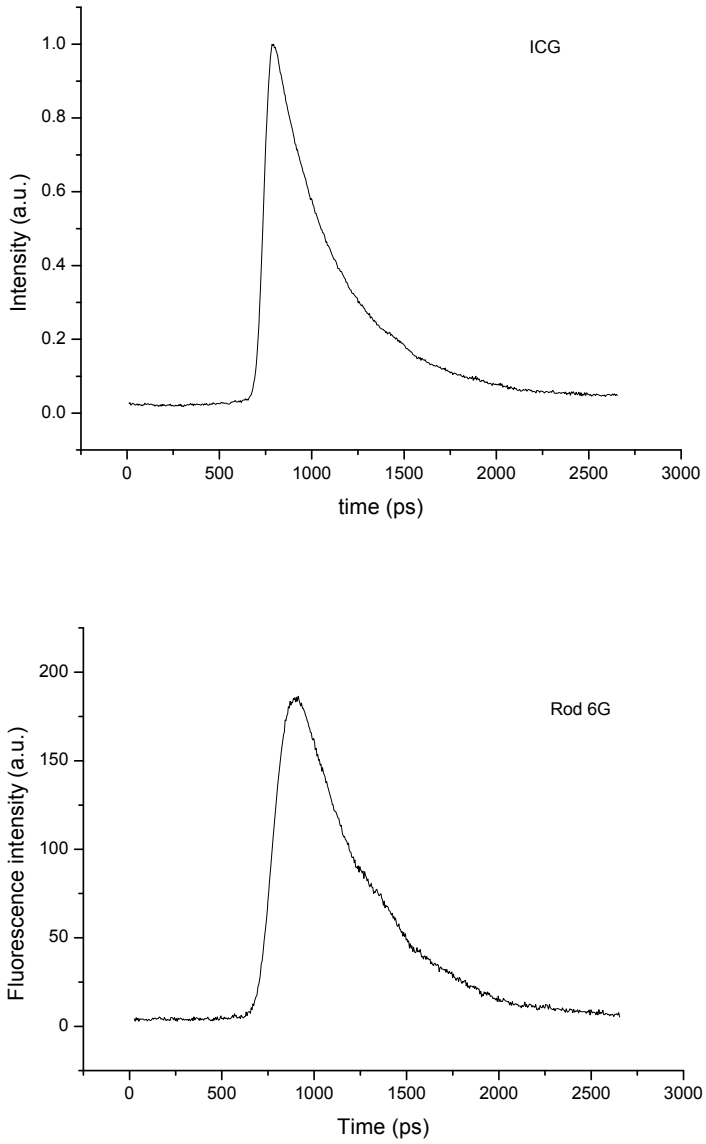


Fig.3 Time- resolved fluorescence spectra of ICG (concentration = 10-3 M, excitation wavelength =800 nm) and Rod 6G (concentration = 10-4 M, excitation wavelength =820 nm)

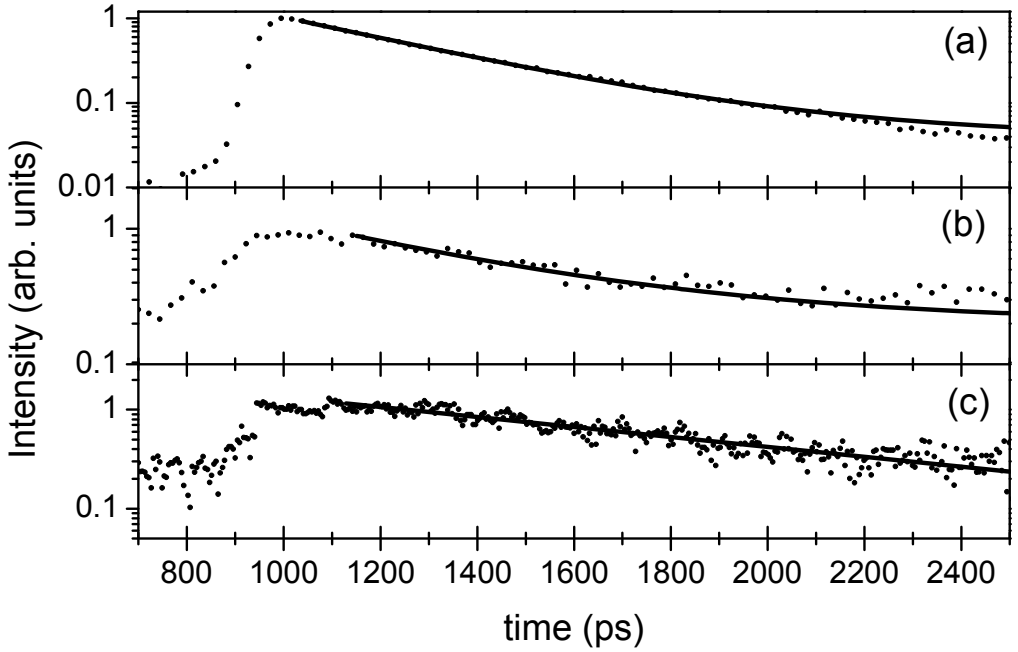


Fig.4 Time-resolved fluorescence spectra of ICG with different Intralipid concentrations. (a) 0% Intralipid, τ=339±4 ps, (b) 5% Intralipid, τ=447±44 ps, (c) 8% Intralipid, τ=602±80 ps

In fig. 4 results of ICG ethanol solutions without and with different Intralipid concentrations are reported. A similar mono-exponential analysis of fluorescence signals have been performed and increasing lifetimes have been obtained for increasing concentration of scattering agents as reported in fig. 4. Similar results for the ICG DMSO solutions have been obtained, when the concentration of scattering agents increases the lifetimes do the same. The results of this analysis are reported in Table 1.

A further analysis of the data has been performed using the above-reported Eq. 2 in order to extract information the reduced scattering coefficients of the scattering media. To do this the optical properties of fluorophore (μ_{fx}, μ_{fm} and Φxm) are to be known. In the present case all the necessary optical information have been obtained by complementary measurements of reflectance and steady-state fluorescence (see ref. 6). The values obtained for the reduced scattering coefficient are reported in Table 2 and shows an increasing linear behavior with the concentration and are in fairly good agreement with μ'_s values obtained with the same apparatus by time-resolved transmittance measurements on Intralipid scattering suspensions (ref. 5 and references therein).

Only in one case (for Rod 6G with no intralipid) we have been able to evidence a double exponential decay (see Table 1), the other measurements showing a mono exponential decay. This is ascribed to a reduction of the signal-to-noise ratio caused by the increased scattering occurring when intralipid is present, which may mask the double exponential decay behaviour.

Sample	τ_1 (ps)	τ_2
ICG	339±4	
ICG with 5% Intralipid	447±44	
ICG with 8% Intralipid	602±80	
Rod 6G	377±2	694±88
Rod 6G with 5% Intralipid	396±40	
Rod 6G with 8% Intralipid	637±82	

Table 1 Numerical results from fitting procedure for ICG and Rod 6G solution with and without Intralipid scattering agents

Intralipid 10% concentration (%) in ICG solution	μ'_s (mm^{-1})
2%	0.130±.003
5%	0.250±.007
8%	0-370±.011

Table 2 **.** Reduced scattering coefficients as a function of Intralipid concentrations.

5. CONCLUSIONS

A preliminary analysis on time-resolved fluorescence signal has been performed using exponential formula and the results of an analytical approach to the fluorescence signal in scattering media. The data here reported for a linearly excited probes (ICG) and a two-photon excited dye (Rod 6G) show that a detailed study on the changes induced on fluorescence lifetime by scattering events using a streak camera could be very useful to test advanced models for fluorescence in scattering media.

6. REFERENCES

[1] "Topics in fluorescence spectroscopy " Vol.1 Ed. J. R. Lakowicz , Plenum Press (1991)
[2] O.O. Abugo, Z. Gryczynski, J.R. Lakowicz "Modulation sensing of fluorophores in tissue: a new approach to drug compliance monitoring" J. Biomed. Optics **4**, 429-442 (1999)
[3] M.S. Patterson, B. W. Pogue « Mathematical model for time-resolved and frequency –domain fluorescence spectroscopy in biological tissue » Appl. Optics. **33**, 1963-1974 (1994)
[4] D. Hattery, V. Chernomordik, M. Loew, I. Gannot, A.Gandjbakhche "Analytical solutions for time-resolved fluorescence lifetime imaging in a turbid medium such as tissue" J. Opt. Soc. Am. A **18**, 1523-1550 (2001); ibid. **20**, (2003)
[5] I. Delfino, M. Lepore, P.L. Indovina "Experimental evaluation of absorption coefficient in scattering media using different solutions to the diffusion equation" Physica Medica XXIV, 134-145 (2002)
[6] M. Lepore, S. Grilli, P.L. Indovina "Fluorescence spectroscopy of scattering media in visible and infrared range" Proc. SPIE n° 4161 (2001)